ISSN (print) 0093-4666 © 2013. Mycotaxon, Ltd. ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/124.101

Volume 124, pp. 101–109 *April–June 2013*

Septoglomus titan, a new fungus in the *Glomeraceae* (*Glomeromycetes*) from Bahia, Brazil

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Abstract — A new fungus of the *Glomeraceae* found in the rhizosphere of *Agave sisalana* is described under the epithet *Septoglomus titan*. It forms large glomerospores, (243–)265 × 325(–400) µm in diameter. They generally are subglobose and have three spore wall layers: a semi-persistent short lived sub-hyaline to yellow-brown outer layer (0.5–1.4 µm thick), an adherent smooth light-yellow to orange-brown middle layer (2.5–5.1 µm), and a laminate smooth thick orange brown to dark red brown innermost layer (12.8–19.2 µm). Spore size and colors of the spore wall layers separate this species from other yellow-brown to dark-brown species.

Key words — *Glomerales*, *Agavaceae*, *Glomus* group A, crop system, rDNA

Introduction

Approximately 230 species of arbuscular mycorrhizal fungi (AMF; *Glomeromycota*) have been described, of which 118 exhibit glomoid spore development (sensu lato; Oehl et al. 2011a, Estrada et al. 2011). Historically, identification of the glomoid species was considered extremely difficult because of the limited morphological characters (Morton 1988), and the molecular approach has been used to solve the hypothetical separation into different genera, families, and orders (Walker & Schüßler 2004, 2007, Schüßler & Walker 2010). For this reason recent taxa from different glomeromycotan groups have been poorly described morphologically (Walker & Schüßler 2004, Schüßler & Walker 2010), including groups with diversified morphological data sets such as *Acaulospora, Kuklospora,* and *Ambispora* (Kaonongbua et al. 2010, Krüger et

al. 2011, Schüßler & Walker 2010). Recently Oehl et al. (2011a,b,c) combined significant morphological characters with concomitant molecular data to reorganize the *Glomeromycota*, especially the groups with glomoid spore development. This newly available morphological data set is being successfully used to identify distinct groups with glomoid spores (Estrada et al. 2011, Furrazola et al. 2011, Goto et al. 2012).

Glomoid species are very commonly found in Brazilian agrosystems (Silva et al. 2007, Goto et al. 2010, Maia et al. 2010, Nobre et al. 2010) with reports of many undescribed species (Carrenho et al. 2010) but with new descriptions rarely available. Studies on AMF diversity in *Agave sisalana* (common name: sisal) crop systems in Bahia State, Brazil, have revealed an undescribed species forming large spores with glomoid development, which we describe here as *Septoglomus titan*.

Material & methods

Study areas, soils sampling and soil parameters

Study areas were in the municipalities of São Domingos located at 12°48′S 41°37′W, and Ourolândia located at 10°58′S 41°05′W, both in the semi-arid region of Bahia State, in the Northeast Brazil. This semi-arid region is characterized by tropical dry climate (type Am of Köppen-Geiger; Kottek et al. 2006), with mean annual temperature of 30°C and precipitation of 400–600 mm. The vegetation was agriculturally managed sisal, a perennial culture of 40-year-old systems, in a Caatinga biome characterized by low tree stratum without a continuous canopy, trees and shrubs with thin stems and small or composite leaves, deciduous in the dry season (Queiroz 2006). The predominant local geology is characterized by a granite-greenstone formed by the Santa Luz complex, Archaean basement, and comprises an assemblage of migmatitic gneisses with subordinate granitoids. The fungus was found in soil samples collected around *Agave sisalana* roots in farms of both municipalities. Soil characteristics in Ourolândia were: pH ($CaCl_2$) = 6.4, organic matter = 13 g dm^3, available P = 18 mg dm^3 and in São Domingos were: pH ($CaCl_2$) = 6.3, organic matter = 32 g dm^3, and available P = 58 mg dm^3.

AMF bait cultures

The native AMF communities were cultured for two cycles (5 months each) with *Sorghum bicolor*, *Brachiaria decumbens,* and *Panicum miliaceum* in 500 mL pots filled with autoclaved sand substrate (250 g per pot) mixed with the natural field soil as AMF inoculum (250 g per pot), at the greenhouse of the Center for Agricultural, Environmental and Biological Research, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, Brazil. Additionally, multiple glomerospores from the new species were separated and used as infective propagules in single species cultures on *S. bicolor*. The new fungus could successfully be propagated in bait cultures together with *Acaulospora scrobiculata*, *Ambispora appendicula, Claroideoglomus etunicatum, Entrophospora infrequens, Funneliformis mosseae, Glomus intraradices, G. sinuosum, Paraglomus brasilianum,* and *P. occultum*. However, no single species cultures were obtained from the fungus. Glomerospores isolated exclusively from the trap cultures were used for morphological and molecular analyses.

Morphological analyses

Spores of the new species were extracted as described in Sieverding (1991). Trap cultures were established according to Goto et al. (2012) modified by using Hoagland's solution without phosphorus. The spores were thereafter mounted in polyvinyl alcohol–lacto–glycerin (PVLG), in PVLG + Melzer's reagent, and in water (Brundrett et al. 1994, Spain 1990). The terminology used is based on recent papers (Furrazola et al. 2011, Goto & Maia 2006, Goto et al. 2012, Estrada et al. 2011)

Molecular analyses

DNA extraction: DNA was extracted from three single spores of the new species. Individual spores on a slide with a drop (5–10 µl) of ultrapure water were crushed with a needle. The resulting material was used directly in the PCR reactions.

Amplification and sequencing: DNA extract was used as template for a semi-nested PCR using the primers ITS3 (White et al. 1990) – 28G2 (Silva et al. 2006) and LR1 (van Tuinen et al. 1998) – 28G2 consecutively. PCR reactions were carried out in a volume of 50 µl, containing 75 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 200 µM each dNTPs, 1 µM of each primer, and 2 units of Taq™ DNA polymerase (Fermentas); cycling parameters were 5 min at 95°C (1 cycle), 45 s at 94°C, 1 min at 55°C, 1 min at 72°C (40 cycles), and a final elongation of 7 min at 72°C following the last cycle. The amplified products were purified with a PureLink PCR Purification Kit (Invitrogen), following the manufacturer's instruction and sequenced. Sequencing was provided by the Human Genome Research Center (São Paulo, Brazil).

Sequence alignment: Querying the National Center for Biotechnology Information databases with the BLASTn program, we verified that the sequences obtained from *S. titan* were affiliated with the *Glomerales* (*Glomeromycota*) before phylogenetic analysis. The AM fungal sequences (partial LSU rRNA) obtained in our laboratory were aligned with other glomeromycotean sequences from the GenBank using the program ClustalX (Larkin et al. 2007) and edited with the BioEdit program (Hall 1999) to obtain a final alignment. The sequences were deposited at GenBank under the accession numbers JQ312667–312669.

Phylogenetic analyses: Maximum parsimony (MP) analysis with 1000 bootstrap replications was performed using the Phylogenetic Analysis Using Parsimony (PAUP) program version 4 (Swofford 2003). Bayesian (two runs over 1×10^6 generations with a burnin value of 2500) and maximum likelihood (1000 bootstrap) analyses were executed, respectively, in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and PhyML (Guindon & Gascuel 2003), launched from Topali 2.5. The model of nucleotide substitution (GTR + G) was estimated using Topali 2.5 (Milne et al. 2004). Sequences from *Claroideoglomus claroideum* and *C. etunicatum* were used as outgroup.

Results

Molecular analyses

Phylogenetic analyses of LSU rRNA gene sequences (Fig. 1) revealed that the new fungus is a *Septoglomus* species (former *Glomus* group Aa3 sensu Oehl et al. 2011a).

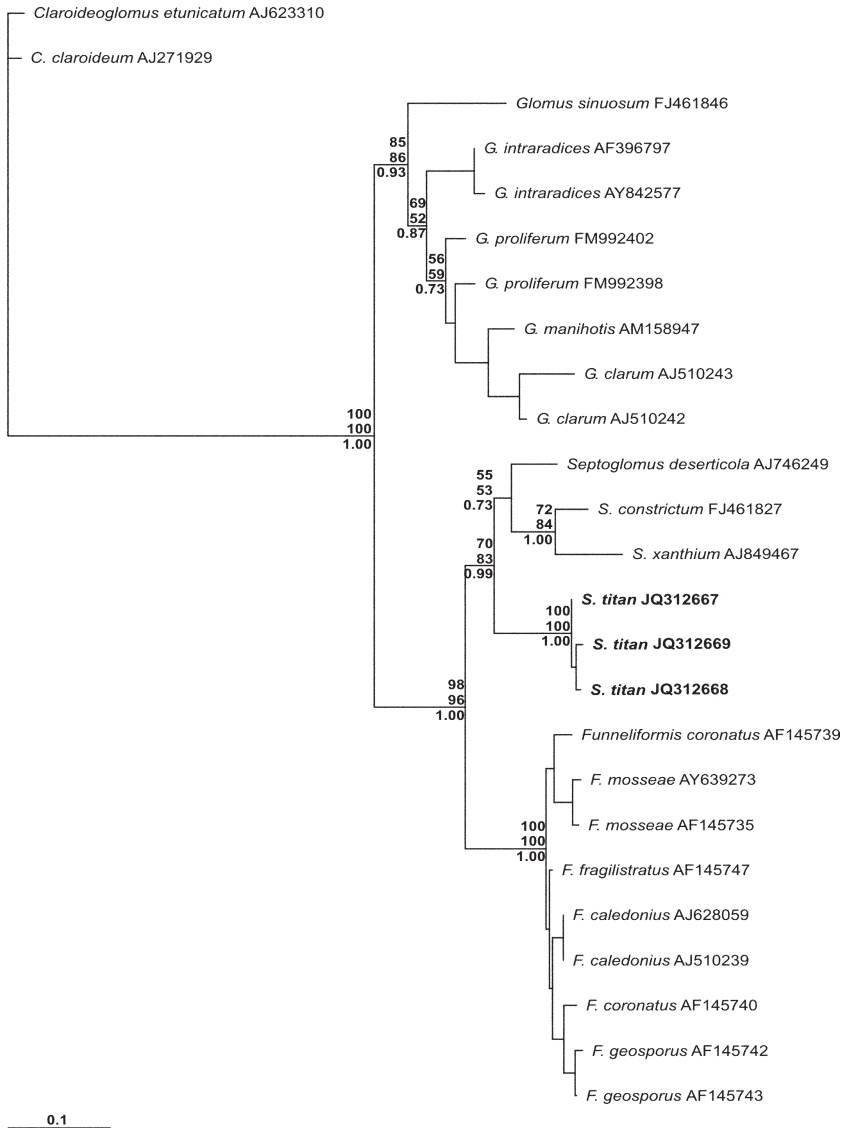
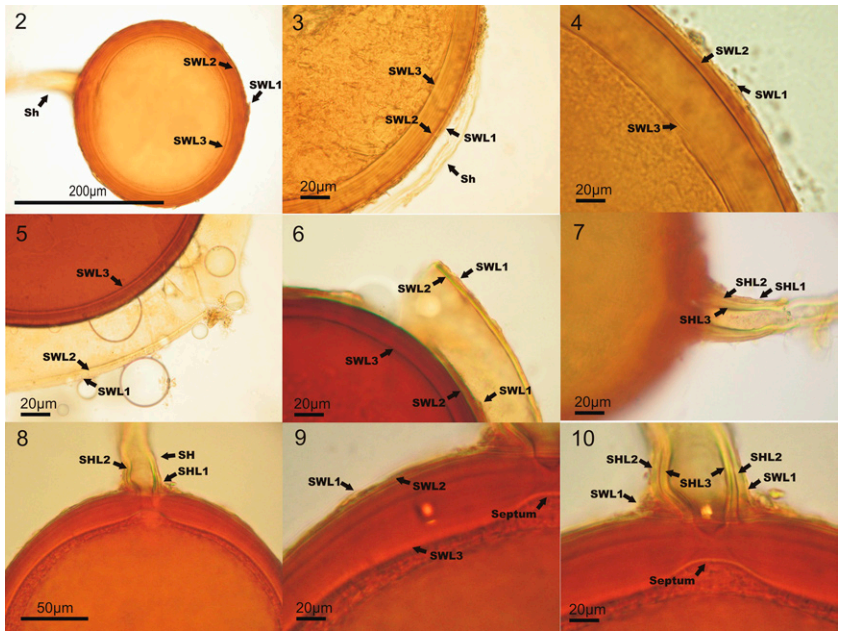


Fig. 1. Phylogenetic tree of the *Glomeraceae* based on LSU rDNA analysis and rooted by *Claroideoglomus claroideum* and *C. etunicatum*. Sequences are labeled with database accession numbers. Support values are from maximum parsimony (MP), maximum likelihood (ML) and bayesian analyses, respectively. *Septoglomus titan* sequences are in bold. Only topologies with ≥50% bootstrap values are shown. (Consistency Index = 0.62; Retention Index = 0.79).



Figs 2–10. *Septoglomus titan*. **2.** Spore formed on subtending hypha (sh) with three spore wall layers (SWL1, SWL2, SWL3) continuous with subtending hyphal wall layers. **3–6**. Spore wall with three layers in young and mature spore (SWL1, SWL2 and SWL3). [3, 4. Young spores with inner layer (SWL3) less pigmented than outer layers (SWL1 and SWL2). 5, 6. Mature spores with inner layer (SWL3) more pigmented than outer layers (SWL1 and SWL2)] **7, 8**. Subtending hyphae slightly funnel-shaped to slightly constricted and all three spore wall layers continuous with subtending hyphal wall (SW). **9, 10**. Detail of septum formed by sublaminae of laminated inner layer (SWL3) typical of *Septoglomus*.

Taxonomic analyses

Septoglomus titan B.T. Goto & G.A. Silva, **sp. nov.** Figs 2–10

MycoBank MB *564321*

Differs from *Septoglomus africanum, S. deserticola,* and *S. xanthium* by its larger spores with 3-layered walls; and from *S. constrictum* by its thicker 3-layered spore walls.

Type: Brazil: Bahia, near Ourolândia, in soil cultivated with *Agave sisalana*, 20.Apr.2011, B.T. Goto, permanent PVLG slides (**Holotype**, URM83528; GenBank JQ312667, JQ312668, JQ312669. **Isotypes**, URM83529; URM83530; Z+ZT Myc 15119).

Etymology: *titan* = in reference to the large spores formed by the new species.

Glomerospores are yellow brown to orange brown when young to dark red brown at maturity, formed terminally on hyphae, and globose to sub-globose (243–)265–375(–400) µm or (rarely) oblong to irregular, (225–)245–285 × 308–325(–400) µm.

Spore wall is (15.3–)17.9–23.0(–25.6) µm thick in total, comprising three smooth layers (SWL1–3). SWL1 is sub-hyaline to light yellow, thin (0.5–1.4 µm) observed in young and mature spores or in the subtending hyphal wall. SWL2 is pigmented, yellow brown to orange brown, unit (2.5–5.1 µm); darker than SWL3 in young spores, SWL2 acquires pigmentation during maturation. The SWL3 is dark yellow to yellow brown in young spores becoming orange brown to dark red brown in mature spores, laminated,12.8–19.2 µm thick. Spore wall layers are continuous with the subtending hyphal wall layers, and the SWL2 and SWL3 pigments continue into the subtending hyphal wall. The three layers do not stain when exposed to Melzer's reagent.

Subtending hypha (sh) generally present, single, straight, cylindrical to constricted, light yellow to orange brown, 17.9–33.2 µm wide (mean = 25.4 µm) at the spore base. The subtending hyphal wall is 7.6–8.9(–10.2) µm thick (mean 8.2 µm) near the spore base, tapering to approximately 2.5–5.2 µm about 100 µm distant from the spore base; occlusion formed by bridging septum arising from sublaminae of SWL3, although the pore at the subtending hypha sometimes appears partly open.

Germination by regrowth of the subtending hypha.

Glomerospore development deduced from spores found in different developmental stages. The sub-hyaline hyphal wall layer differentiates into sub-hyaline to yellow, evanescent (SWL1) and unified (SWL2) spore wall layers after which the laminate layer 3 differentiates with increasing number of developing sublaminae and becomes pigmented. Finally, the pore is not closed by introverted thickening of SWL3 but by a bridging septum arising from the laminate wall layer at the spore base.

Arbuscular mycorrhiza formation associated with roots of *Sorghum bicolor* (L.) Moench.

Distribution — Currently known only from agricultural soils mainly cultivated with *Agave sisalana* Perrine (*Agavaceae*) in Ourolândia and São Domingos, Bahia State (Brazil).

Additional collections examined: **BRAZIL**: **Bahia**, near São Domingos, in soil cultivated with *Agave sisalana*, UFRN1895 UFRN1896 UFRN1897.

Discussion

Sequence analyses of the partial LSU rRNA region confirm with high support that the new species represents a *Septoglomus* that differs from the four other described *Septoglomus* species (Fig. 1). The morphology of the subtending hyphal structure (Figs 3–8) also supports the new species in *Septoglomus* as circumscribed by Oehl et al (2011a).

Septoglomus africanum (Błaszk. & Kovács) Sieverd. et al., *S. deserticola* (Trappe et al.) G.A. Silva et al., and *S. xanthium* (Błaszk. et al.) G.A. Silva et al.

differ from *S. titan* by their smaller spores with 2-layered walls [*S. africanum* (60–)87(–125) µm (Błaszkowski et al. 2010); *S. deserticola* 47–54(–115) × 38–52(–102) µm (Trappe et al. 1984); and *S. xanthium* 20–55 × 45–100 µm (Błaszkowski et al. 2004)]. Although *Septoglomus constrictum* (Trappe) Sieverd. et al. also has large (150–330 µm) spores, it differs from *S. titan* by its thinner, 2-layered spore walls with a total thickness of 7–15 µm (Trappe 1977, Oehl et al. 2011a).

Septoglomus titan is easily distinguished from all other glomoid non-*Septoglomus* species by spore wall structure. A few dark species such as *Glomus tenebrosum* (Thaxt.) S.M. Berch and *Funneliformis geosporus* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler resemble *S. titan*. *Glomus tenebrosum* has spores with only two spore wall layers (Thaxter 1922, Trappe 1977, Walker 1982). *Funneliformis geosporus* has spores with three wall layers, but the inner layer is flexible and thin (<1.0 µm), whereas in *S. titan* the SWL3 is laminated and thick.

Goto et al. (2012) noted that the spore wall of *G. trufemii* B.T. Goto et al. was one of the thickest (7.4–15.5 µm) among the glomoid-spored glomeromycotan species, but *S. titan* has even thicker spore walls.

Acknowledgements

The authors acknowledge Dr. Janusz Błaszkowski (Department of Plant Protection, West Pomeranian University of Technology, Szczecin, Poland) and Dr. Fritz Oehl (Agroscope Reckenholz-Tänikon ART, Zurich, Switzerland) for reviewing the manuscript and making helpful comments and suggestions and appreciate the corrections by Dr. Shaun Pennycook (Nomenclature Editor) and suggestions by Dr. Lorelei Norvell (Editor-in-Chief). This work was supported by: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) through Protax (Programa de Capacitação em Taxonomia); PPBio (Programa de Biodiversidade do Semiárido) and INCT-HV (Herbário Virtual da Flora e dos Fungos); FACEPE (Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco); UFRN (Programa de Pós-Graduação em Sistemática e Evolução); UFPE (Programa de Pós-Graduação em Biologia de Fungos) and CAPES-PNPD (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Programa Nacional de Pós-Doutorado). The Graduate Program in Microbiologia Agrícola da Universidade Federal do Recôncavo da Bahia is acknowledged for providing technical support.

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